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An Unusual Pathway for the Synthesis of Adenosine Triphosphate by the Purine-requiring Organism *Artemia salina**

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SUMMARY

Brine shrimp are incapable of synthesizing purines *de novo* and seemingly have a complex nucleotide metabolism involving conversion of stored diguanosine 5′,5‴-(P^1,P^4-tetraphosphate) into ATP. When nucleosides uniformly labeled with ^{14}C are administered to the organism, its ATP shows an unusual labeling pattern. When [*U*-^{14}C]adenosine is used as a precursor, the purine moiety is primarily incorporated into ATP, whereas when brine shrimp are exposed to [*U*-^{14}C]guanosine, the ribose component is preferentially incorporated into ATP. This selective labeling of the purine and ribose moieties of ATP occurs at early time intervals after exposure to the labeled nucleoside, whereas AMP and ADP have [^{14}C]purine to [^{14}C]ribose ratios similar to that of the original nucleoside administered. Thus there appears to be a route from adenosine and guanosine to ATP that does not proceed through AMP and ADP. A pathway is proposed involving the direct participation of diguanosine 5′,5‴-(P^1,P^4-tetraphosphate) as an intermediate.

During early development, the brine shrimp *Artemia salina* contains large amounts of guanine derivatives, principally diguanosine 5′,5‴-(P^1,P^4-tetraphosphate),[1] but only very small amounts of adenine compounds (1). Furthermore, the organism is incapable of synthesizing purines *de novo*, and after hatching it converts guanine compounds to adenine compounds with Gp_4G,[2] apparently serving as a direct precursor of ATP (2). As a consequence of these observations, we have focused our attention on the mechanism by which brine shrimp convert guanine-containing precursors to ATP.

We observed that when nauplii of *A. salina* are given uniformly labeled guanosine or adenosine for short periods of time, the ratio of [^{14}C]purine to [^{14}C]ribose in ATP shows an extensive disproportionality, and the selective utilization of the individual components of the precursor is dictated by the nitrogenous base. Further, this unusual labeling pattern of ATP in nauplii occurs despite the fact that AMP and ADP have purine to ribose ratios similar to that of the nucleoside administered.

EXPERIMENTAL PROCEDURE

Methods

Nauplii were harvested after hatching as described by Finamore and Clegg (3).

Determination of [^{14}C]Purine to [^{14}C]Ribose Ratios—Labeled purine nucleosides were purified by chromatography on Dowex 50-H^+ (4) and deacidified by adsorbing, washing, and eluting from charcoal as described below. The purine to ribose ratios of the [*U*-^{14}C]nucleosides (Schwarz-Mann, Amersham-Searle) were verified in the following manner. Aliquots were hydrolyzed in 0.5 N HCl for 30 min at 100°, diluted to 0.1 N HCl, and applied to a Dowex 50-H^+ column (1 × 3 cm). The ribose moiety passed through the resin in the void volume, and the purine component was obtained by a stepwise elution with 50 ml each of 0.25, 0.5, 1.0, and 2.0 N HCl (4). Radioisotopes in aqueous solution were counted in a solution containing 2.6 g of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]-thiophene (Packard Instrument Co.) per liter of methyl Cellosolve-toluene (3:5, v/v) using a Packard Tri-Carb liquid scintillation spectrometer. The [^{14}C]purine to [^{14}C]ribose ratios were found to be between 0.89 and 1.0 in all cases.

Artemia were incubated with the purified labeled compound in Petri dishes (6 × 1.5 cm) containing 10 ml of artificial seawater fortified with penicillin (1000 units per ml). After various periods of incubation, the brine shrimp were collected and washed on cloth filter supports.

Preparation of Acid-soluble Fractions—Nauplii were homogenized in ice-cold 0.5 N $HClO_4$ in a ground glass homogenizer. The homogenate was centrifuged at 25,000 × *g* for 20 min, and the supernatant was applied to charcoal and eluted with ethanol, water, and ammonia (2:2:1, v/v). The acid-soluble nucleotides were separated by DEAE-cellulose (Whatman DE23) chromatography (2, 5). The nucleotides were eluted from the cellulose with a 3000-ml linear gradient of NH_4HCO_3 (0.002 to 0.3 M at pH 8.6) and identified by A_{280}:A_{260} ratios as well as order of elution. Radioactivity was quantitated by scintillation spectrometry throughout the elution sequence and was also helpful in the isolation of the nucleotide components.

The [^{14}C]purine to [^{14}C]ribose ratios of the isolated compounds

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[1] This compound originally was referred to as P^1,P^4-diguanosine-5′-tetraphosphate, but Dr. K. L. Loening, Director of Nomenclature, *Chemical Abstracts Service*, and member of IUPAC, suggests diguanosine 5′,5‴-(P^1,P^4-tetraphosphate) as a more accurate name.

[2] The abbreviation used is: Gp_4G, diguanosine 5′,5‴-(P^1,P^4-tetraphosphate).

were determined by acid hydrolysis (0.5 N HCl, 100°, 30 min) and subsequent Dowex 50-H^+ chromatography (4). We consider to be biologically significant only deviations in [^{14}C]purine to [^{14}C]-ribose ratios of 2-fold or more.

RESULTS

As shown in Table I, when 1.5-day-old nauplii were incubated in the presence of [*U*-^{14}C]guanosine (purine to ribose ratio = 0.89) for various periods of time, adenine as well as guanine nucleotides were labeled. The purine and ribose moieties of the labeled precursor appear to have been incorporated together in most of the nucleotides at incubation times of 15 to 60 min. The most notable exception is ATP, which at 15 and 30 min had ratios of [^{14}C]purine to [^{14}C]ribose of 0.27 and 0.41, respectively. At 45 and 60 min, however, the ratio approached that of the administered precursor, suggesting the establishment of an equilibrium among the adenine nucleotides. It should be noted that, at all time intervals examined, AMP and ADP did not show any disproportionate labeling pattern.

Table II shows the [^{14}C]purine to [^{14}C]ribose ratios of adenine nucleotides when [*U*-^{14}C]adenosine was administered to nauplii. [^{14}C]Purine to [^{14}C]ribose ratios were not determined for guanine nucleotides because of the reluctance of the organism to convert adenine precursors to guanine components. In contrast to the results obtained by incubation with [*U*-^{14}C]guanosine, it can be seen that, with [*U*-^{14}C]adenosine as precursor, the purine component of ATP and not the ribose component was used preferentially at all incubation times studied. Furthermore, both AMP and ADP reflected the [^{14}C]purine to [^{14}C]ribose ratios of the administered precursor.

Earlier studies showed that brine shrimp can utilize exogenously supplied purine nucleosides as purine nucleotide precursors throughout their life cycle (6). In addition, we found that both guanosine and adenosine were incorporated into the acid-soluble pool of the organism and that their [^{14}C]purine to [^{14}C]ribose ratios were the same as that of the original isotopically labeled nucleosides. Furthermore, analysis of the distribution of acid-soluble counts at various times after a 30-min pulse with [^{3}H]guanosine followed by a chase with unlabeled guanosine for 24 hours showed that over 95% of the ^{3}H found in the medium was accounted for as guanosine, with no radioactive nucleotides being present. This suggests that administered purine nucleosides pass into and out of the acid-soluble pool and indicates that transport of nucleosides into the pool takes place with conservation of the glycosidic linkage.

DISCUSSION

In an earlier report from this laboratory (2) we indicated that the concentration of diguanosine tetraphosphate in nauplii of *Artemia* decreases progressively with time after hatching. Furthermore we observed that, of all the nucleotides present in the organism, only ATP shows an increase in concentration concomitant with the decrease in Gp_4G. On the basis of these observations, we suggested that Gp_4G serves as a direct precursor of ATP, with its guanine moiety being converted to adenine and its pyrophosphate bonds being conserved in the transition. As will be pointed out below, only a slight modification of this idea is necessary to account for our recent observations.

The present study shows that when either guanosine or adenosine is administered to the purine-requiring organism, the [^{14}C]-purine to [^{14}C]ribose ratio in AMP or ADP does not deviate from the predicted value. However, the same ratio in ATP reveals a disproportionate labeling pattern that is apparently dictated by the nitrogenous base. These results indicate that an unusual

TABLE I

[^{14}C]Purine to [^{14}C]ribose ratios of acid-soluble nucleotides after incubation of nauplii[a] with [U-^{14}C]guanosine[b]

Nucleotide	Incubation time							
	15 min		30 min		45 min		60 min	
	Total[c]	[^{14}C] purine/[^{14}C] ribose ratio	Total[c]	[^{14}C] purine/[^{14}C] ribose ratio	Total[c]	[^{14}C] purine/[^{14}C] ribose ratio	Total[c]	[^{14}C] purine/[^{14}C] ribose ratio
GMP	14.4	0.74	20.0	0.81	28.2	0.92	54.0	1.0
GDP	X[d]	X	X	X	2.2	1.0	1.1	0.89
GTP	3.5	0.95	6.9	1.2	40.0	0.98	31.5	0.91
Gp_4G	X	X	X	X	0.9	0.83	1.0	0.97
AMP	1.1	0.84	2.2	0.59	6.2	0.77	3.3	0.84
ADP	1.7	0.93	X	X	6.8	0.77	1.0	0.86
ATP	2.6	0.27	5.5	0.41	30.6	0.65	55.5	0.85

[a] Nauplii, 1.5 days old, were collected and placed in four 6-cm Petri dishes (0.5 g wet weight per dish) with 4.5×10^7 cpm [*U*-^{14}C]-guanosine (260 mCi per mmole, ICN) in 10 ml of incubation medium. Acid-soluble fractions were prepared at the times indicated, applied to charcoal, eluted, concentrated, and applied to DEAE-cellulose columns. The nucleotides were eluted, and the appropriate fractions were isolated and hydrolyzed.

[b] [*U*-^{14}C]Guanosine purine to ribose ratio = 0.89.

[c] Percentage of total counts in medium $\times 10^3$.

[d] X indicates insignificant number of counts incorporated.

TABLE II

[^{14}C]Purine to [^{14}C]ribose ratios of acid-soluble nucleotides after incubation of nauplii[a] with [U-^{14}C]adenosine[b]

Nucleotide	Incubation time							
	15 min		30 min		45 min		60 min	
	Total[c]	[^{14}C] purine/[^{14}C] ribose ratio	Total[c]	[^{14}C] purine/[^{14}C] ribose ratio	Total[c]	[^{14}C] purine/[^{14}C] ribose ratio	Total[c]	[^{14}C] purine/[^{14}C] ribose ratio
AMP	32	0.79	38	1.5	64	1.4	62	1.1
ADP	48	1.3	38	1.6	144	1.2	186	1.6
ATP	80	9.5	57	7.3	160	6.0	180	5.7

[a] Nauplii, 1.5 days old, were incubated with 5.8×10^6 cpm [*U*-^{14}C]adenosine (200 mCi per mmole, ICN) for the times indicated. The various fractions were prepared as described in Table I.

[b] [*U*-^{14}C]Adenosine purine to ribose ratio = 0.95.

[c] Percentage of total counts in medium $\times 10^3$.

pathway exists for the synthesis of ATP, which does not require the participation of either AMP or ADP.

In order to explain the participation of diguanosine tetraphosphate in the synthesis of ATP and the unusual labeling pattern of this compound, we propose that the following reactions are operative in *Artemia*. In both pathways, Gp_4G plays a central role in the synthesis of ATP.

Fate of Adenosine—The asterisk denotes position of the ^{14}C label and the brackets signify possible minor reactions.

$$\underset{\text{(Ado)}}{\overset{*}{\text{Ade-Rib}}} \rightleftarrows \underset{\text{(AMP)}}{\overset{* \quad *}{\text{Ade-Rib-P}}} \rightleftarrows \underset{\text{(ADP)}}{\overset{* \quad *}{\text{Ade-Rib-PP}}}[\rightleftarrows \underset{\text{(ATP)}}{\overset{* \quad *}{\text{Ade-Rib-PPP}}}] \quad (1)$$

$$\text{Ade-Rib-P} + PP_i \rightleftarrows \text{Ade} + \text{PP-Rib-P} \quad (2)$$
(AMP) (phosphoribosyl pyrophosphate)

$$\text{Ade} + \text{Gua-Rib-PPPP-Rib-Gua} \rightleftarrows \text{Ade-Rib-PPPP-Rib-Gua} + \text{Gua} \quad (3)$$
(Gp_4G) (Ap_4G)

$$\text{Ade-Rib-PPP-Rib-Gua} \rightarrow \text{Ade-Rib-PPP} + \text{Gua-Rib-P} \quad (4)$$
(Ap_4G) (ATP) (GMP)

On the basis of the similarities of the [14C]purine to [14C]ribose ratios of Ado, AMP, and ADP, we presume a normal "kinase" system is present in the organism (Step 1). Once some AMP is formed, adenine phosphoribosyltransferase (EC 2.4.2.7) in the presence of PP_i catalyzes the production of free labeled adenine and phosphoribosyl pyrophosphate (Step 2). In support of this contention is the observation that extracts of *Artemia* nauplii contain a potent adenine phosphoribosyltransferase but do not possess significant quantities of hypoxanthine (guanine) phosphoribosyltransferase (EC 2.4.2.6) activity.[3] Once free adenine is formed, we postulate that a purine exchange reaction with Gp_4G occurs, so that a guanine moiety is released from Gp_4G and is replaced by a labeled adenine to produce Ap_4G (Step 3). The formation of such a hybrid molecule is not unusual; for example, Zamecnik and his colleagues (7) described the synthesis of Ap_4G by *Escherichia coli* lysyl-tRNA synthetase preparations. Following the formation of Ap_4G, we visualize an asymmetric cleavage of the molecule to yield unlabeled GMP and ATP labeled only in the purine moiety (Step 4). Although the existence of this enzyme is speculative at this time, Warner and Finamore (8) have isolated a diguanosinetetraphosphatase (EC 3.6.1.17) from *Artemia* embryos that hydrolyzes Gp_4G to yield equimolar quantities of GTP and GMP.

Another point to be made concerning this pathway is that for at least 1 hour the ATP that is formed from adenosine retains its high [14C]purine to [14C]ribose ratio. An explanation for this observation is presented below.

Fate of Guanosine—The asterisk denotes position of the 14C label and the brackets signify possible minor reactions.

$$\underset{\text{(IMP)}}{\text{Hyp-Rib-P}} \rightarrow \underset{\text{(AMP)}}{\text{Ade-Rib-P}} \rightleftarrows \underset{\text{(ADP)}}{\text{Ade-Rib-PP}}[\rightleftarrows \underset{\text{(ATP)}}{\text{Ade-Rib-PPP}}]$$

$$\underset{\text{(Guo)}}{\text{Gua-Rib}} \rightleftarrows \underset{\text{(GMP)}}{\text{Gua-Rib-P}} \rightleftarrows \underset{\text{(GDP)}}{\text{Gua-Rib-PP}} \rightleftarrows \underset{\text{(GTP)}}{\text{Gua-Rib-PPP}} \quad (1)$$

$$2\ \underset{\text{(GTP)}}{\text{Gua-Rib-PPP}} \rightleftarrows \underset{\text{(Gp_4G)}}{\text{Gua-Rib-PPPP-Rib-Gua}} + PP_i \quad (2)$$

$$\underset{\text{(Gp_4G)}}{\text{Gua-Rib-PPPP-Rib-Gua}} + \text{Ade} \rightleftarrows \underset{\text{(Ap_4G)}}{\text{Ade-Rib-PPPP-Rib-Gua}} + \text{Gua} \quad (3)$$

$$\underset{\text{(Ap_4G)}}{\text{Ade-Rib-PPPP-Rib-Gua}} \rightarrow \underset{\text{(ATP)}}{\text{Ade-Rib-PPP}} + \underset{\text{(GMP)}}{\text{Gua-Rib-P}} \quad (4)$$

Since the [14C]purine to [14C]ribose ratios of guanosine, GMP, GDP, and GTP are similar, we assume a normal kinase system exists for the transformation of guanosine to its phosphorylated derivatives (Step 1). In the next step of our proposed sequence, we do not consider the role of hypoxanthine (guanine) phosphoribosyltransferase to be important because of its sluggish activity in extracts of nauplii.[3] Rather we believe the production of GTP allows the diguanosine tetraphosphate synthetase enzyme described by Warner and Beers (9) to come into play, producing labeled Gp_4G with the liberation of PP_i (Step 2). This synthetic step permits both guanine and ribose moieties of Gp_4G to be labeled equally (see Table I). Steps 3 and 4 are then visualized to be the same purine exchange and cleavage sequence as outlined above, the over-all result being that with guanosine as precursor the newly synthesized ATP is labeled mostly in the ribose moiety.

It should be noted that within a short period of time the [14C]purine to [14C]ribose ratio of the ATP formed from guanosine returns to the same level found in the mono- and diphosphates, whereas the [14C]purine to [14C]ribose ratio of the ATP formed from adenosine does not. This difference can be explained by the fact that the organism preferentially converts guanine-containing nucleosides or nucleotides or both to their adenine-containing counterparts. Thus radioactive adenine becomes available via the adenine phosphoribosyltransferase reaction. Some labeled adenine can then participate in the purine exchange reaction with Gp_4G, resulting in the formation of ATP with a [14C]purine to [14C]ribose ratio approaching unity. Since *Artemia* does not readily convert adenine compounds to guanine compounds, the [14C]purine to [14C]ribose ratio of the ATP formed from adenosine remains at an unusually high level.

Both pathways for the synthesis of ATP account not only for the peculiar distribution of label within the ATP molecule but also for the contribution Gp_4G makes to the entire process. In addition, the scheme allows for the inverse relationship between the concentrations of ATP and diguanosine tetraphosphate that occurs during development (2).

We are currently searching for the enzymes responsible for the proposed purine exchange with diguanosine tetraphosphate and the asymmetrical pyrophosphohydrolase that cleaves Ap_4G.

[3] E. A. Hiss and F. J. Finamore, unpublished results.

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